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Functional heterogeneity in the process of T lymphocyte activation; barium blocks several modes of T cell activation, but spares a functionally unique subset of PHA-activable T cells

L. T. PEÇANHA & G. A. DOS REIS* *Carlos Chagas Filho Institute of Biophysics, and *Department of Immunology, Institute of Microbiology, Federal University of Rio de Janeiro, Brazil*

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SUMMARY

The effects of the alkaline earth divalent cation Barium (Ba^{2+}) were studied in in-vitro murine polyclonal T cell activation induced with a panel of T cell mitogens consisting of the plant mitogens concanavalin A (ConA), jacalin and phytohaemagglutinin (PHA), a mitogenic anti-Thy1 monoclonal antibody (MoAb), and an anti-murine CD3 MoAb combined with phorbol ester. All modes of T cell activation, except PHA-induced mitogenesis, were blocked in a reversible and dose-related manner by Ba^{2+}. Blockade was evident only if Ba^{2+} was added within the first 6 h of stimulation, was totally reversed in a competitive fashion by addition of Ca^{2+} to the medium, and selectively affected interleukin 2 (IL-2) production, without interfering with expression of IL-2 receptor light chains, nor with late IL-2-dependent activated T cell growth. On the other hand, PHA-induced responses stimulated by optimal mitogen doses were resistant to the effects of Ba^{2+}. Ba^{2+}-resistance of PHA responses was due to IL-2-dependent activation and growth of a Ba^{2+}-resistant T cell subset since: (i) limiting dilution analysis demonstrated that this PHA response had a much lower precursor cell frequency than control PHA responses; (ii) proliferation was blocked by anti-IL2 agents, such as cyclosporin A and anti-IL-2 receptor light chain MoAbs, which were much less effective in blocking control PHA responses. Thus, pharmacological use of Ba^{2+} reveals the existence of a pathway of T cell activation, induced by PHA, with differential interleukin requirements.

Keywords T cell activation barium cyclosporin A interleukin 2 phytohaemagglutinin

INTRODUCTION

The use of plant mitogens has been of great help in elucidating several aspects of T lymphocyte immunobiology, including the sequence of membrane, cytoplasmic and nuclear events involved in the process of T cell activation. Initial studies led to the notion of a universal role for the T cell-derived lymphokine interleukin 2 (IL-2), as the endogenous mitogenic signal for T lymphocytes (Smith, 1980), and of Ca^{2+} ions as the critical second messenger initiating T cell activation (Freedman, 1979; Tsien, Pozzan & Rink, 1982). However, recent studies done on IL-4-dependent helper T cell lines (Lichtman, Kurt-Jones & Abbas, 1987), together with analysis of Ca^{2+} responses in different modes of T cell activation (Holter *et al.*, 1986; Hu-Li *et al.*, 1987; Ho *et al.*, 1987) have challenged the universality of both concepts. These studies have suggested that there are distinct modes of T cell activation, some of which could be Ca^{2+}-independent, and some might not use IL-2 as a critical activation/growth factor. Little is known about the nature of the physiological situations in which these unusual T cell responses are triggered and what the functional consequences are of T cell activation through these alternative mechanisms. In an attempt to uncover some complexities in the process of T cell activation triggered by natural plant mitogens, or by mitogenic anti-T cell monoclonal antibodies (MoAbs), we have screened the effects of the divalent cation Ba^{2+} on T cell activation induced *in vitro* by a variety of T cell stimuli. Ba^{2+} blocks several modes of polyclonal T cell activation, apparently by interfering with an early, Ca^{2+}-dependent step related to IL-2 production in responding T lymphocytes. Here we describe a T cell activation response, which is selectively induced by phytohaemagglutinin (PHA) and which is resistant to blockade by Ba^{2+}. This response has a differential interleukin requirement for growth, when compared to control PHA responses.

Correspondence: Dr George A. Dos Reis, Departamento de Imunologia, Instituto de Microbiologia da UFRJ, Centro de Ciências da Sáude, Bloco I, Cidade Universitária, Ilha do Fundão, 21941, Rio de Janeiro, Brazil.

MATERIALS AND METHODS

Animals

Balb/c mice of both sexes, aged 6–8 weeks, were used throughout these studies.

Responding cells

Splenocytes from normal Balb/c mice were passed through a nylon wool column, as described by Julius, Simpson & Herzenberg (1973). The resulting nylon wool non-adherent cells (NWNAC) were used as a source of responding T cells throughout these studies. When accessory cells were needed, whole splenocyte populations from normal Balb/c mice were irradiated (2300 rad) and used.

All cultures were performed in RPMI 1640 medium (Grand Island Biological Co., NY) supplemented with bicarbonate, 2-mercaptoethnaol (5×10^{-5} M), gentamycin (10 μg/ml), L-glutamine (2 mM), Hepes buffer (10 mM) and 5% fetal calf serum (FCS). Splenic T cells ($2–4 \times 10^5$) were cultured in 0·2 ml of culture medium in flat-bottom, 96-well microtitre plates (Limbro, Handen, CT) for 48–72 h at 37°C, and at 7% CO_2 in a humid atmosphere. Six to 18 h before harvesting, cultures were pulsed with 1 μCi of tritiated thymidine (^{3}H-TdR; 6·7 Ci/mmol; New England Nuclear Corp., Boston, MA). Cultures were terminated by collection through a Mini-Mash harvesting device (M.A. Bioproducts; Walkersville, MD) onto fibreglass filters. The amount of ^{3}H-TdR incorporated into cellular DNA was assessed by standard liquid scintillation spectroscopy. All cultures were done in triplicate. The standard error of the mean rarely exceeded 10% of the mean value and is omitted for simplicity. Results are expressed as mean counts per minute of triplicate cultures or as the difference between stimulated and control cultures.

Assessment of T cell activation parameters

Splenic T cells (2×10^6) were cultured with mitogen in 1 ml of culture medium in 24-well culture vessels (Limbro), for 20 h at 37°C, 7% CO_2, and saturated water environment. The IL-2 bioactivity of these supernatants was assessed by their ability to support the growth of the IL-2-dependent CTLL-2 T cell line. The non-adherent cells were recovered, passed through a Ficoll-Hypaque separation medium (Pharmacia Inc., Piscataway, NJ) for 20 min at 400 *g*. Viable cells were collected at the interface, washed and assayed for surface expression of IL-2 receptor light chains, as described elsewhere (Dos Reis, Nóbrega & Paes de Carvalho, 1986).

In addition, viable cells recovered from bulk cultures after 72 h of concanavalin A (ConA) addition were washed with methyl-alpha-D-mannopyranoside (20 mg/ml) to remove residual ConA and recultured (2×10^4) in the absence of mitogens, with human recombinant IL-2 (rec IL-2; Cetus Laboratories) for 24 h in 96-well (5×10^3) microtitre plates, in the absence or in the presence of barium. CTLL-2 cells were also used in these tests. Proliferation was assessed by ^{3}H-TdR uptake.

Limiting dilution and double reciprocal analysis

Splenic T cells (NWNAC) were cultured in 20 independent wells of a 96-well microtitre plate, with a fixed number (1×10^5) of irradiated accessory cells, with mitogen and with 10% v/v of an IL-2-enriched supernatant obtained by PMA stimulation of the EL-4 thymoma. Control cultures were set up for each cell number in 4 wells and did not receive mitogen.

After 5 days, proliferation was assessed by ^{3}H-TdR uptake. Individual cultures were considered positive when ^{3}H-TdR incorporation exceeded the upper limit of incorporation in the control cultures (a value given by the sum of the mean ct/min of control wells plus three standard deviations). Double reciprocal analysis of the barium inhibitory effect in the presence of several extracellular calcium concentrations was performed as previously described (Dos Reis *et al.*, 1986).

Reagents

The following T cell mitogens were employed: ConA (Pharmacia, Uppsala, Sweden), PHA (Wellcome Diagnostics, Dartford, England), jacalin, prepared as described (Bunn-Moreno & Campos-Neto, 1981), and donated by Dr Marlene Bunn-Moreno, Federal University of Rio de Janeiro. A rat MoAb directed to the murine Thy 1.2 molecule, MoAb G7 (Gunter, Malek & Shevach, 1984), and a combination of hamster anti-murine CD3 ε chain, MoAb 145-2C11, and PMA 10 ng/ml (Leo *et al.*, 1987), were also used. These MoAbs were donated by Dr Ethan M. Shevach, NIH, MD. Cyclosporin A (CyA, Sandoz, Inc., Hanover, NJ), was dissolved in ethanol at 1 mg/ml and then serially diluted in culture medium to give the desired dosages. Stock solutions were stored protected from light. CyA was a generous gift form Dr Manoel Barral Neto, Federal University of Bahia, Brazil.

RESULTS

Screening of several modes of T cell activation by sensitivity to blockade with barium

Splenic NWNAC were stimulated *in vitro* with a panel of T cell mitogens consisting of the plant mitogens ConA, jacalin and PHA, a mitogenic anti-Thy1 MoAb (G7), and a combination of an anti-CD3 MoAb (hamster anti-CD3 ε chain MoAb 145-2C11) and phorbol ester. Cultures were done in the absence and in the presence of 2·0 mM Ba^{2+}. Initial experiments revealed that Ba^{2+} was the only alkaline earth divalent cation with a potent suppressive effect on in-vitro ConA-induced mitogenesis. The suppressive effect of Ba^{2+} was dose-related, and did not result from toxicity, since T cells pre-cultured with Ba^{2+} resumed normal mitogenic responses after washing (data not shown). As shown in Table 1, Ba^{2+} blocked all tested modes of polyclonal T cell activation, except that induced by an optimal mitogenic dose of PHA. All mitogens were added at optimal stimulating doses, as assessed in previous dose–response curves. Although not shown in Table 1, the IC_{50} of the Ba^{2+} inhibitory effect was fairly similar for all mitogens tested, within a given experiment.

Barium-induced blockade affects an early step in T cell activation and can be reversed by Ca^{2+} ions

The timing of blockade of T cell activation by Ba^{2+} was investigated in kinetic experiments using anti-Thy1 MoAb G7 as stimulus. As shown in Table 2, Ba^{2+} blocks G7-induced T cell activation, only if added within the first 6 h after stimulation, but not 8 or more hours after G7 addition to the cultures. These results indicate that Ba^{2+} blockade is not the result of toxicity and, in addition, establishes that the target of Ba^{2+} action must be an early activation step. This notion was confirmed when T cell activation was dissected, and separately analysed. As shown in Fig. 1, Ba^{2+} selectively blocks IL-2 production from T cells stimulated by either G7 or ConA.

On the other hand, as shown in Fig. 1, expression of IL-2 receptor light chains is not blocked by Ba^{2+}. Also, IL2-dependent activated T cell growth is left untouched (or even

Table 1. Screening for Ba^{2+}-resistant polyclonal T cell activation

T cell stimulus*	^{3}H-TdR incorporation (ct/min)† in the presence of Medium	Ba^{2+}‡	% suppression
anti-CD3/PMA	103833	3929	96·2
anti-Thy1	34635	491	98·6
Con A	107083	2791	97·4
Jacalin	86500	7576	91·2
PHA	109095	95741	12·2

* Splenic NWNAC (2×10^5) cultured for a total of 72 h with the following reagents: hamster anti-murine CD3 MoAb 145-2C11, 1 μg/ml, plus PMA, 10 ng/ml; rat anti-murine Thy1.2 MoAb G7, 1:500 ascites; ConA, 2·5 μg/ml; jacalin, 30 μg/ml; PHA, 2·0 μg/ml. Unstimulated T cells gave 1497 ct/min.

† Proliferation was assessed by ^{3}H-TdR uptake after 72 h in culture.

‡ $BaCl_2$, 2·0 mM, was added to the cultures.

Table 2. Kinetics of Ba^{2+}-induced blockade of T cell mitogenesis

Time of Ba^{2+} addition* (hours after G7 addition)	^{3}H-TdR incorporation (ct/min)	% suppression
0	8622	67·9
2	9272	65·5
4	10616	60·4
6	14686	45·3
8	22740	15·3
10	20757	22·7
22	27660	0
No Ba^{2+} added	26834	0

* Splenic NWNAC (3×10^5) were stimulated at time zero with MoAb G7, 1:1500 ascites. $BaCl_2$, 4·0 mM was added either simultaneously, or after the indicated time intervals. Proliferation of unstimulated T cells was 824 ct/min. Proliferation was assessed by ^{3}H-TdR uptake after 48 h in culture.

enhanced, in some experiments) by maximally inhibitory doses of Ba^{2+} (Fig. 1).

Barium-induced blockade of T cell activation can be reversed by addition of excess Ca^{2+} ions to the extracellular medium, as shown in Fig. 2. The figure also shows a double reciprocal analysis of the inhibitory effect of Ba^{2+}, done at several extracellular doses of added Ca^{2+} ions. A typical common *y*-axis intercept appeared in two independent experiments, indicating a competitive antagonism between Ba^{2+} and Ca^{2+} ions in the control of ConA-induced T cell activation. On the other hand, exogenously applied rec IL-2 was unable to reverse Ba^{2+} blockade (not shown) suggesting that, besides IL-2 receptor light chain expression some unidentified early Ba^{2+}-sensitive process is also required for T cell responsiveness to IL-2. Taken together, these results indicate that Ba^{2+} blocks several modes of T cell activation, by interfering with an early Ca^{2+}-dependent step, related to (but not solely) IL-2 release.

Analysis of a T lymphocyte activation pathway resistant to blockade by Ba^{2+} and selectively induced by PHA

The results presented in Fig. 3 illustrate the previous observation (Table 1) that PHA-induced T cell activation is resistant to Ba^{2+} blockade. PHA-induced mitogenesis is actually enhanced by Ba^{2+}, and is only marginally reduced by doses of Ba^{2+} which are high enough to induce irreversible effects on ConA-induced T cell responses. Ba^{2+} blocks ConA responses at any ConA dose tested. At low PHA doses, Ba^{2+} blocks this response, but this blocking effect disappears at optimal and supraoptimal mitogenic doses of PHA (not shown).

Limiting dilution analysis of PHA responses, in the presence and in the absence of Ba^{2+}, was performed. In the absence of Ba^{2+}, the curve reveals a component of suppressive activity, which is lost upon further dilution. In the presence of Ba^{2+}, no suppressive component is apparent by diluting T cells, and the precursor frequency is about 20% of the highest precursor frequency detected in control PHA responses. Thus, Ba^{2+}-resistant T cells comprise a fraction of the total PHA-activable T cell pool in normal spleen. This sub-population does not correlate with $L3T4^+$ or $Lyt2^+$ subsets, since both subsets respond to PHA, and both subsets show Ba^{2+}-resistant proliferative responses (not shown).

To test whether this T cell response done in the presence of Ba^{2+} characterizes a distinctive activation pathway, its sensitivity to anti-IL2 agents *in vitro* was compared with that of control PHA responses. Anti-IL2 agents included CyA and MoAbs directed to the murine IL-2 receptor light chains (7D4 and 3C7). Representative results are shown in Fig. 4 and Table 3. Figure 4 shows dose–response curves of the effect of CyA on T cell activation induced by ConA, PHA and PHA + Ba^{2+}. As shown, PHA-induced T cell activation is resistant to increasing doses of CyA, and is not further affected by 500 ng/ml CyA, a dose which might already be toxic for murine ConA-induced responses, since exogenous IL-2 fails to reverse blockade at this CyA concentration (not shown). On the other hand, ConA-induced responses are markedly suppressed, in a dose-related fashion, by this same concentration range of CyA. More important, as shown in Fig. 4, Ba^{2+}-resistant PHA T cell response of the same magnitude as the control PHA response, is markedly suppressed by the same doses of CyA which blocked ConA responses, but which had no effect on control PHA responses. In other separate experiments CyA induced a partial blockade of control PHA responses, never exceeding 50% of the control response even at low doses, but this partial blockade could not be further enhanced by increasing CyA dosage (data not shown). On the other hand, Ba^{2+}-resistant PHA responses are always highly sensitive to blockade, even by small CyA doses.

Table 3 shows that addition of a mixture of MoAbs 7D4 and 3C7, directed to IL-2 receptor light chains, to PHA-activated T cell cultures had only a marginal effect on cell proliferation (6·0% suppression of control PHA response). However, addition of the same mixture to the same NWNAC stimulated by PHA in the presence of Ba^{2+}, led to a pronounced blockade (72·2% suppression of the Ba^{2+}-resistant response) of the resulting proliferative response.

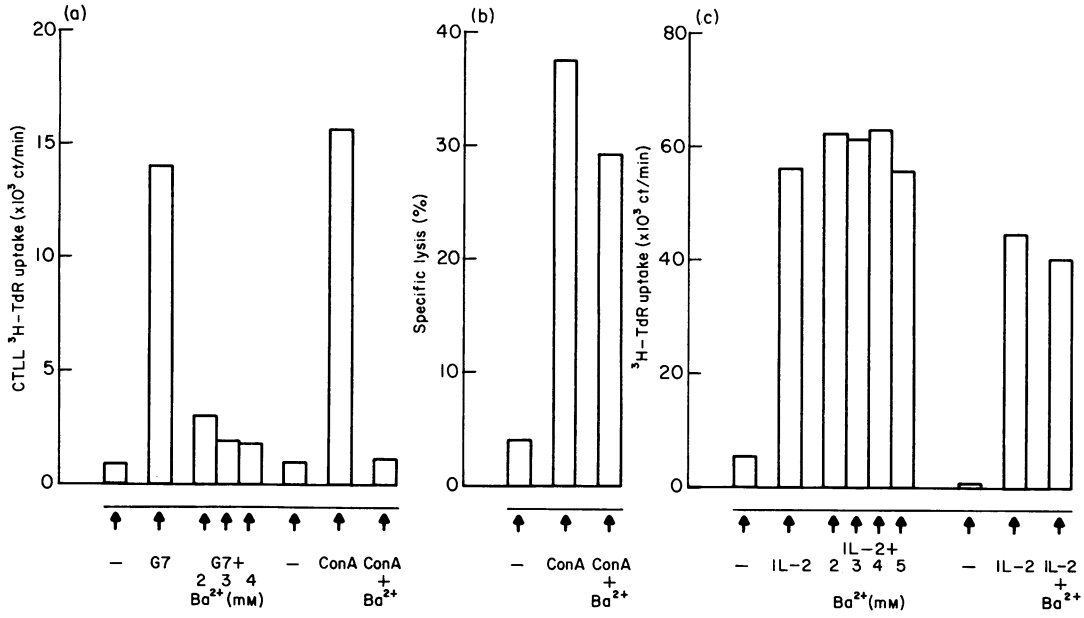

Fig. 1. Effect of Ba^{2+} on T cell activation parameters. (a) IL-2 production. Splenic NWNAC (2×10^6) were cultured with either MoAb G7 (1/500) or ConA (2·5 μg/ml) for 20 h in the presence or in the absence of the indicated doses of Ba^{2+} (4 mM when not indicated). Supernatants were collected, stored, and tested for IL-2 bioactivity on CTLL-2 cells. (b) IL-2 receptor expression. Splenic NWNAC (2×10^6) were cultured with or without ConA (2·5 μg/ml) for 20 h, in the presence or in the absence of Ba^{2+} (4 mM). The resulting viable cells were collected on a density gradient, and IL-2 receptor expression was evaluated by an anti-IL-2 receptor MoAb plus complement assay (see text for details). Results express mean percent specific lysis of a typical experiment. (c) IL-2-driven activated T cell growth. Left panel: CTLL-2 cells were cultured with rIL-2 (50 U/well) in the absence or in the presence of the indicated doses of Ba^{2+}. Proliferation was assessed after 24 h in culture. Right panel: ConA-activated T cell blasts were cultured in the presence of methyl-alpha-D-mannopyranoside (20 mg/ml) with rIL-2 (50 U/well) in the presence or in the absence of Ba^{2+}, 4 mM. Proliferation was assessed after 24 h in culture.

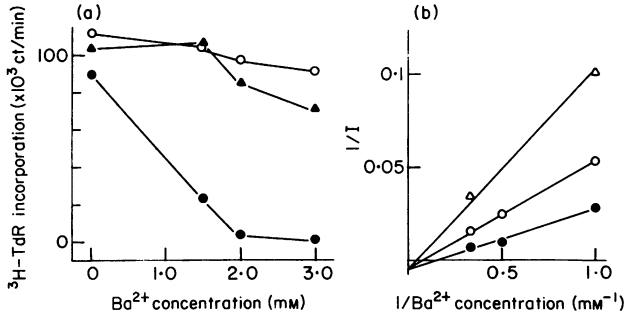

Fig. 2. Reversal of Ba^{2+}-induced blockade by exogenously applied Ca^{2+}. (a) Splenic NWNAC (2×10^5) were cultured with ConA (2·5 μg/ml) in the presence of the indicated doses of Ba^{2+}, and in the absence (●), or in the presence of $CaCl_2$, 1·5 mM (▲), or 3·0 mM (○). Unstimulated T cells gave 1497 ct/min. Proliferation was assessed by ^{3}H-TdR after 72 h in culture: (b) A similar experiment, showing a double-reciprocal analysis of Ca^{2+}-reversal of Ba^{2+}-induced blockade of ConA responses. ● Absence of Ca^{2+}, ○ 2·0 mM $CaCl_2$, △ 3·0 mM $CaCl_2$. Unstimulated T cells gave 1047 ct/min, ConA-stimulated cultures gave 103 531 ct/min in medium alone, 74 632 ct/min in 2 mM Ca^{2+}, and 65 100 ct/min in 3 mM Ca^{2+}.

The data presented in Fig. 4 and Table 3 suggest that: (i) murine T cell activation by PHA, done in the absence of Ba^{2+}, is IL-2-independent to a large extent; (ii) Ba^{2+}-resistant T cell activation induced by PHA, on the other hand, critically relies on IL-2 as an activation and/or growth promoting factor, in the same way as occurs with ConA responses.

DISCUSSION

In the present study, we have shown that Ba^{2+} ions can be pharmacologically used to characterize a unique murine T lymphocyte activation pathway, selectively induced by PHA, and with distinctive interleukin requirements.

The mechanism of Ba^{2+}-induced blockade we noted on several modes of T cell activation is unknown. The rationale for using Ba^{2+} as a blocker of T cell activation was that Ba^{2+} permeates T cell Ca^{2+} channels (Kuno *et al.*, 1986) and thus could interfere with Ca^{2+}-dependent processes which depend on a Ca^{2+}-influx. However, the evidence of a Ca^{2+} antagonism role for Ba^{2+} is only circumstantial, at present. Effects of Ba^{2+} on early biochemical events of T cell activation are being currently investigated. Blockade of T cell activation by Ba^{2+} is completely reversed, in a competitive fashion, by adding Ca^{2+} ions to the medium. Moreover, Ba^{2+} selectively blocks IL-2 production, without interfering with expression of IL-2 receptor light chains, nor with late IL-2-mediated proliferation of the activated T cell. These findings are in agreement with the notion of Ca^{2+}

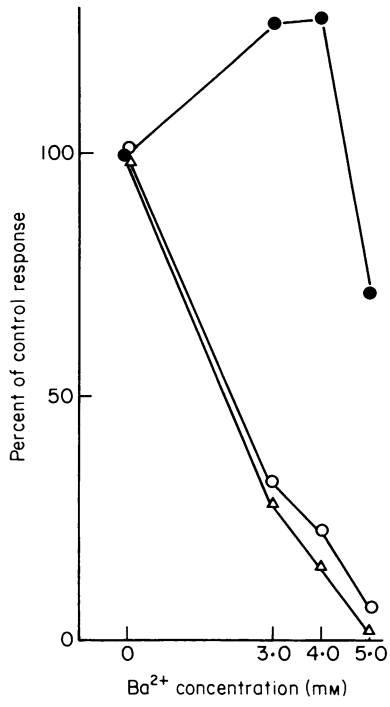

Fig. 3. Barium resistance of PHA-induced response. Splenic NWNAC (4×10^5) were cultured with ConA, 2·0 μg/ml (○), anti-Thy1 MoAb G7, 1:500 ascites (△), or PHA, 4·0 μg/ml (●), in the presence of the indicated doses of $BaCl_2$. Proliferation was assessed by ^{3}H-TdR after 48 h in culture. Unstimulated T cells gave 5328 ct/min. Control ConA response was 152355 ct/min; control G7 response was 75859 ct/min; control PHA response was 129012 ct/min.

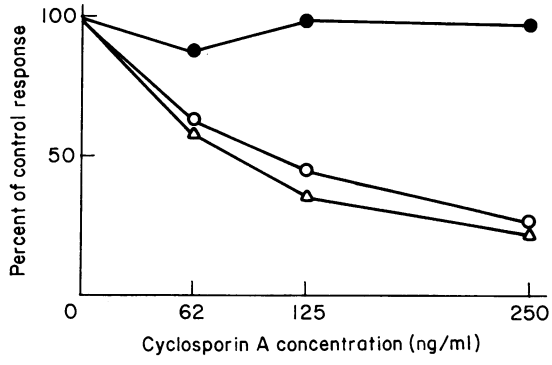

Fig. 4. Differential sensitivity of PHA responses to CyA. Splenic NWNAC (2×10^5) were cultured with ConA, 2·5 μg/ml (△), or PHA, 2·0 μg/ml, in the absence (●) or presence (○) of Ba^{2+}, 2 mM and at the indicated doses of CyA. Maximal dose of ethanol present was 0·1%, and had no effect on control T cell responses. Proliferation was assessed by ^{3}H-TdR uptake after 72 h in culture. Unstimulated T cells gave 566 ct/min. Control T cell responses were: 116227 ct/min for ConA; 78412 ct/min for PHA; 123999 ct/min for PHA plus Ba^{2+}.

Table 3. Effect of anti-IL2 receptor MoAbs on PHA responses

	^{3}H-TdR incorporation (delta ct/min)* in	
MoAbs†	Control PHA	Ba^{2+}-resistant PHA
None	52044	65917
7D4/3C7	48909	18350
% suppression	6·0	72·2

* Splenic NWNAC (4×10^5) were cultured in the absence or in the presence of 1·0 μg/ml PHA, with or without $BaCl_2$, 4 mM. Proliferation was assessed by ^{3}H-TdR uptake after 48 h in culture. Unstimulated cultures gave 7512 ct/min.

† Anti-IL2 receptor light chain MoAbs 7D4 and 3C7 were added at the start of culture, at 1:200 ascites, each.

antagonism, since previous studies established that IL-2 production, but not expression of IL2 receptor light chains, nor IL-2-mediated T cell growth, is Ca^{2+}-dependent (Mills *et al.*, 1985a, b). Because several recent studies indicated that certain modes of T cell activation could proceed without a rapid rise in cytoplasmic free Ca^{2+} concentration, we used Ba^{2+} as a screening agent for detecting T cell activation pathways with a greatly reduced or absent early Ca^{2+} requirement. Within a panel of five well-known T cell mitogens, only PHA-induced T cell responses were resistant to blockade by Ba^{2+}. One possibility is that PHA induces a much more potent activation signal and that Ba^{2+} is unable to shut off this signal delivered to the T cell.

However, in the present study, we gathered strong evidence for another possibility, namely, that T cells responding to PHA in the presence of Ba^{2+} are functionally distinct from control PHA-responsive T cells.

At present, we have no evidence for the nature of the T cell membrane proteins responsible for transducing activation signals in this situation. We propose that Ba^{2+} acts by sparing special T cell precursors which can initiate their activation sequence, in spite of dampened Ca^{2+} signals.

That Ba^{2+}-resistant responses are mounted by a circumscribed T cell sub-population within the PHA-activable cell pool, was found in limiting dilution experiments. The frequency of the Ba^{2+}-resistant population was estimated to be 45% of the PHA-responsive pool of lower frequency, or, perhaps more accurately, as 20% of the total PHA-responsive pool, when the suppressive component of the dilution curve is disregarded. Ba^{2+}-resistant T cells contain both L3T4$^+$ and Lyt2$^+$ precursors, although resting Lyt2$^+$ cells seem more enriched in Ba^{2+}-resistant precursors (data not shown). Ba^{2+}-resistant blasts are hyperresponsive to IL-2, and L3T4$^+$ cells are the IL-2-hyperresponsive cells in this system (not shown). Hyperresponsiveness to IL-2 helps to explain why Ba^{2+}-resistant T cell responses are of the same magnitude as control PHA responses after three days, in spite of a lower precursor frequency.

In-vitro T cell responses induced by PHA are IL-2-independent to a great extent (Table 3 and Fig. 4), as judged by lack of blockade with either CyA or anti-IL2 receptor MoAbs. ConA responses of comparable magnitude were severely reduced by CyA (Fig. 4) or by anti IL-2 receptor MoAbs (not shown). Previous studies had already shown a relative IL-2 independence of PHA-activable T cells (Malek *et al.*, 1984; Mire-Sluis *et al.*, 1987). On the other hand, Ba^{2+}-resistant T cell growth

induced by PHA is markedly sensitive to blockade with CyA or anti-IL-2 receptor MoAbs. This finding indicates that IL-2 is a critical requirement for the Ba^{2+}-resistant pathway, and supports the view that Ba^{2+}-resistant T cells comprise a distinct and circumscribed set of T cell precursors within the PHA-activatable cell pool.

Within the PHA response, the nature of the lymphokine responsible for Ba^{2+}-sensitive, IL-2-independent T cell growth is unknown. However, it is not IL-4, since anti-IL-4 MoAb 11B11 blocks Ba^{2+}-resistant, but not control PHA responses (Peçanha & Dos Reis, 1988). One important aspect to be further investigated in our studies, is the marked sensitivity of Ba^{2+}-resistant T cells to CyA. One general conclusion from data in the literature, is that CyA selectively blocks Ca^{2+}-dependent, but not Ca^{2+}-independent lymphocyte activation (Klaus & Clisholm, 1986), and this notion disagrees with Ba^{2+}-resistant T cell activation being Ca^{2+}-independent. We noted that much higher doses of Ba^{2+} actually block completely the PHA response (not shown). Therefore, Ba^{2+}-resistance should be regarded as relative, rather than absolute resistance. It is possible that Ba^{2+}-resistant T cells are, in fact, much less dependent on Ca^{2+}, but not Ca^{2+}-independent at all, for activation. The possibility that Ba^{2+}-resistant T cells use only internal release of Ca^{2+} for activation should be considered. Also, Ba^{2+} resistance could distinguish between virgin and memory T cell activation. Preliminary data indicate that density-separated large and small T cells strikingly differ in Ba^{2+} sensitivity. Thus, use of Ba^{2+} may help characterize differences in activation processes of T cells from distinct differentiation stages.

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